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Temporal pattern of jasmonate-induced alterations in gene expression of barley leaves

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Abstract. Leaf tissues of barley (*Hordeum vulgare* L. cv. Salome) respond to methyl jasmonate (JaMe) treatment with a characteristic pattern of gene expression. Jasmonate-induced proteins (JIPs), such as leaf thionins (*jip15* gene product) and ribosome-inactivating proteins (*jip60* gene product), rapidly accumulate. Their genes are transiently transcriptionally activated, as shown here by the determination of in-vitro transcription rates in run-off assays. In contrast to *jip* genes, expression of photosynthetic genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcS* gene product) and a type III light-harvesting chlorophyll-*a*/*b*-binding protein (LHCP; *lhbC1* gene product), for example, was rapidly down-regulated in JaMe-treated barley leaves. Despite decreasing rates of *rbcS* and *lhbC1* gene transcription, their transcripts were maintained in JaMe-treated leaf tissues for at least 36 h. Only at a later stage, was there a decline in the levels of *rbcS* and *lhbC1*, but not *jip*, transcripts, suggesting a selective destabilization of photosynthetic mRNAs in JaMe-treated leaf tissues.

Key words: Gene expression – *Hordeum* – mRNA destabilization – Methyl jasmonate – Transcriptional control

Introduction

Jasmonic acid and its derivatives, such as the methyl ester, methyl jasmonate (JaMe), represent a novel class of ubiquitous plant growth regulators (reviewed by Ryan 1992; Sembdner and Parthier 1993). Jasmonates are involved in various physiological processes (Sembdner and Parthier 1993). In almost all cases studied, symptoms induced by JaMe coincided with the appearance of novel proteins designated jasmonate-induced proteins (JIPs Weidhase et al. 1987; see Reinbothe et al. 1994b, for a compilation of JaMe-responsive genes).

In barley, at least four groups of prominent *jip* genes have thus far been identified by cDNA cloning procedures (Andresen et al. 1992; Becker and Apel 1992) and Northern hybridization with gene-specific probes (Reinbothe et al. 1992a,b, 1994a). Based on the comparison of deduced amino-acid sequences, putative functions could be inferred for all of the encoded polypeptides with the exception of the 23-kDa (Andresen et al. 1992) and 37-kDa JIPs (Leopold et al. 1996), which share no similarity with other known protein sequences found in the data banks.

One barley JIP, JIP6, which is synthesized as a 15-kDa precursor (JIP15; Andresen et al. 1992), was found to belong to the group of leaf thionins, fungitoxic molecules localized in the plant cell wall and in the vacuole (Bohlmann et al. 1988). Another JIP, JIP60 (Becker and Apel 1992), was identified to be a ribosome-inactivating protein involved in the general down-regulation of the protein biosynthetic machinery in long-term JaMe-treated or long-term-stressed leaf tissues (Chaudhry et al. 1994; Reinbothe et al. 1994c). Finally, some *jip* transcripts seem to encode "late embryogenesis abundant" (LEA) proteins (Reinbothe et al. 1992a,b, 1994a) that are thought to mitigate the physico-chemical changes encountered in cells of vegetative and generative tissues during cytosolic dehydration (Dure et al. 1989; Dure 1993).

Another observation pertains to the negative effect exerted by JaMe on the expression of plastid- and nuclear-encoded chloroplast proteins (Reinbothe et al. 1993a–c). It has been suggested that amino acids released from the degradation of prominent plastid proteins, such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), might be used for rapid JIP formation (Weidhase et al. 1987).

Dedicated to Professor Dr. Andreas Sievers, Bonn, on the occasion of his 65th birthday

Abbreviations: JaMe = jasmonic acid methyl ester (methyl jasmonate); JIP(s) = jasmonate-induced protein(s); Rubisco = ribulose-1,5-bisphosphate carboxylase/oxygenase

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To further characterize this multitude of observations, we determined the temporal pattern of expression of *jip* and photosynthetic genes in JaMe-treated barley leaf tissues.

Materials and methods

Plant material. Seeds of barley (*Hordeum vulgare* L. cv. Salome; Martin-Luther-Universität Halle, Experimentelle Station Seehausen, Germany) were germinated on moist vermiculite under continuous white light illumination (ca. 30 $W \cdot m^{-2}$, provided by fluorescent bulbs) for 7 d. Leaves were cut from the seedlings, and 5-cm segments beginning 1 cm from the leaf tip were placed onto either water or a 45-μM aqueous solution of JaMe (Firmenich, Geneva, Switzerland) for various periods. For inhibitor experiments, leaf segments were incubated with either water or JaMe for 24 or 36 h before cordycepin addition to a final concentration of 500 $\mu g \cdot ml^{-1}$.

Nuclear run-off transcription assay. Nuclei were isolated according to Willmitzer and Wagner (1981) and Chappell and Hahlbrock (1984). With this method, 25 g of freshly harvested barley leaves yielded approx. 10^9 nuclei. The yield of intact nuclei, as assessed by counting their number on an equal-leaf-number basis, dropped over the time of JaMe incubation, and, after 72 h, accounted for approx. 30% of the initial level recovered from freshly harvested leaves. In contrast, intact nuclei could be isolated with approx. twofold higher yields from leaf segments that had been incubated with water in the absence of JaMe for 72 h.

In 60-μl reaction volumes, 10^7 nuclei were incubated with 80 μCi (2.96 MBq) α-[^{32}P]UTP (111 $TBq \cdot mmol^{-1}$; NEN DuPont de Nemours, Dreieich, Germany) for various time intervals (Chappell and Hahlbrock 1984). Radiolabeled nuclear run-off transcripts synthesized during a 30-min incubation in 600-μl reaction volumes were hybridized with nitrocellulose filters that contained cDNA inserts encoding the following proteins: JIP23, JIP15 and JIP60 of barley (Andresen et al. 1992; Becker and Apel 1992), β-tubulin of pea (Liaud et al. 1992), actin of soybean (Shah et al. 1982), a small subunit of Rubisco of *Lemna gibba* (*rbcS*) (Stiekema et al. 1983), and a type III light-harvesting chlorophyll-*a*/*b*-binding protein of barley (*lhbC1*; Brandt et al. 1992). After hybridization under standard conditions (Sambrook et al. 1989), the filters were washed three times for 10 min each with 1 × SSC (0.15 M sodium citrate, 0.015 M sodium chloride)/0.1% (w/v) SDS, first at 42 °C and then at 62 °C, and three times for 10 min each with a solution containing 0.1 × SSC/0.1% SDS at room temperature. Radiolabeled run-off transcripts hybridizing to the filter-bound cDNA inserts were detected by autoradiography.

Preparation and Northern hybridization of RNA. Total RNA purified from the various leaf samples by extraction with phenol/chloroform/isoamyl alcohol and precipitation with LiCl (Müller-Uri et al. 1988) was separated electrophoretically, as described previously (Reinbothe et al. 1993b). Nitrocellulose filters (BA-S 85; Schleicher & Schuell, Dassel, Germany) containing the different RNAs were hybridized with *rbcS*, *lhbC1* and *jip60* cDNA inserts, as decribed previously (Reinbothe et al. 1993b). Nucleic acid hybrids were detected by autoradiography and their radioactivities quantitated with a liquid scintillation counter (see above). Unless stated otherwise, transcript levels were expressed as percentages of the initial levels found in freshly harvested barley leaves. The half-life times of the *rbcS* and *lhbC1* transcripts were calculated by plotting linear regressions of the data shown in Figs. 2 and 3.

Other techniques. The SDS-PAGE in exponential 11–20% polyacrylamide gradients was performed according to Laemmli (1970). Western blotting to determine the amount of JIP60 protein in the different leaf samples was performed according to Towbin et al. (1979), using the goat anti-rabbit IgG, anti-goat IgG alkaline-phosphatase system. Ribonucleoprotein material was isolated from the various leaf samples by differential centrifugation of crude cell homogenates, followed by $MgCl_2$ precipitation and sucrose density gradient fractionation (Reinbothe et al. 1993b). Polysome profiles were recorded at 254 nm during harvest of the gradients, and the P/T ratios as a measure of the amount of ribosomes in polysomes and thus of the overall rate of translation initiation were calculated as described previously (Reinbothe et al. 1994c).

Results

Determination of transcription rates in nuclear run-off assays. Labeled run-off transcripts formed by an equal number of nuclei from the various leaf samples were hybridized with restriction-endonuclease-digested cDNAs encoding two abundant barley JIPs immobilized on nitrocellulose membranes. The probes included a member of the family of the 23-kDa JIPs (Andresen et al. 1992) and the precursor of a leaf thionin (JIP15; Andresen et al. 1992).

As shown in Fig. 1A, both the 23-kDa JIP and thionin cDNA inserts (lanes a and b, respectively) strongly hybridized with RNAs synthesized in nuclei isolated from JaMe-treated leaf tissues. In both cases, *jip* gene transcription was first detectable 4 h after exposing leaf tissues to JaMe. Maximum rates of transcription were observed approx. 8 h after the onset of JaMe treatment. Thereafter, transcription of the thionin (*jip15*) and *jip23* genes began to decrease and was no longer detectable after 48 h of JaMe treatment. In water-treated leaf tissues, a low level of *jip23* and *jip15* gene transcription could be observed but only after 48 h, presumably reflecting a late wound response (Fig. 1A).

In contrast to *jip* genes, photosynthetic genes, such as *rbcS* encoding the small subunit of Rubisco, and *lhbC1* encoding a type III light-harvesting complex (LHC) protein, were already transcribed in freshly harvested barley leaves (Fig. 1B). Their transcription, as well as that of genes encoding actin and tubulin, was maintained in water-treated leaf tissues for at least 24 h, after which transcription was reduced to a low but still detectable level (Fig. 1B). Methyl jasmonate appeared to promote this decline, as seen by the rapid decrease in the rates of transcription of the *rbcS* and *lhbC1* genes and the delayed effect on the transcription of the actin and tubulin genes (Fig. 1B).

Quantification of RNA levels by Northern hybridization. To determine whether the observed changes in transcription of the above-mentioned photosynthetic genes caused equivalent changes in message abundances, Northern blots were hybridized with *rbcS*- and *lhbC1*-specific cDNA inserts identical to those used for nuclear run-off experiments. Nucleic acid hybrids were detected by autoradiography and quantified by either scanning the optical density of the signals on the films or directly by liquid scintillation counting. Both methods of analysis provided identical results.

As summarized in Fig. 2, freshly harvested barley leaves abundantly expressed both the *rbcS* and *lhbC1* transcripts. These initial transcript levels were main-

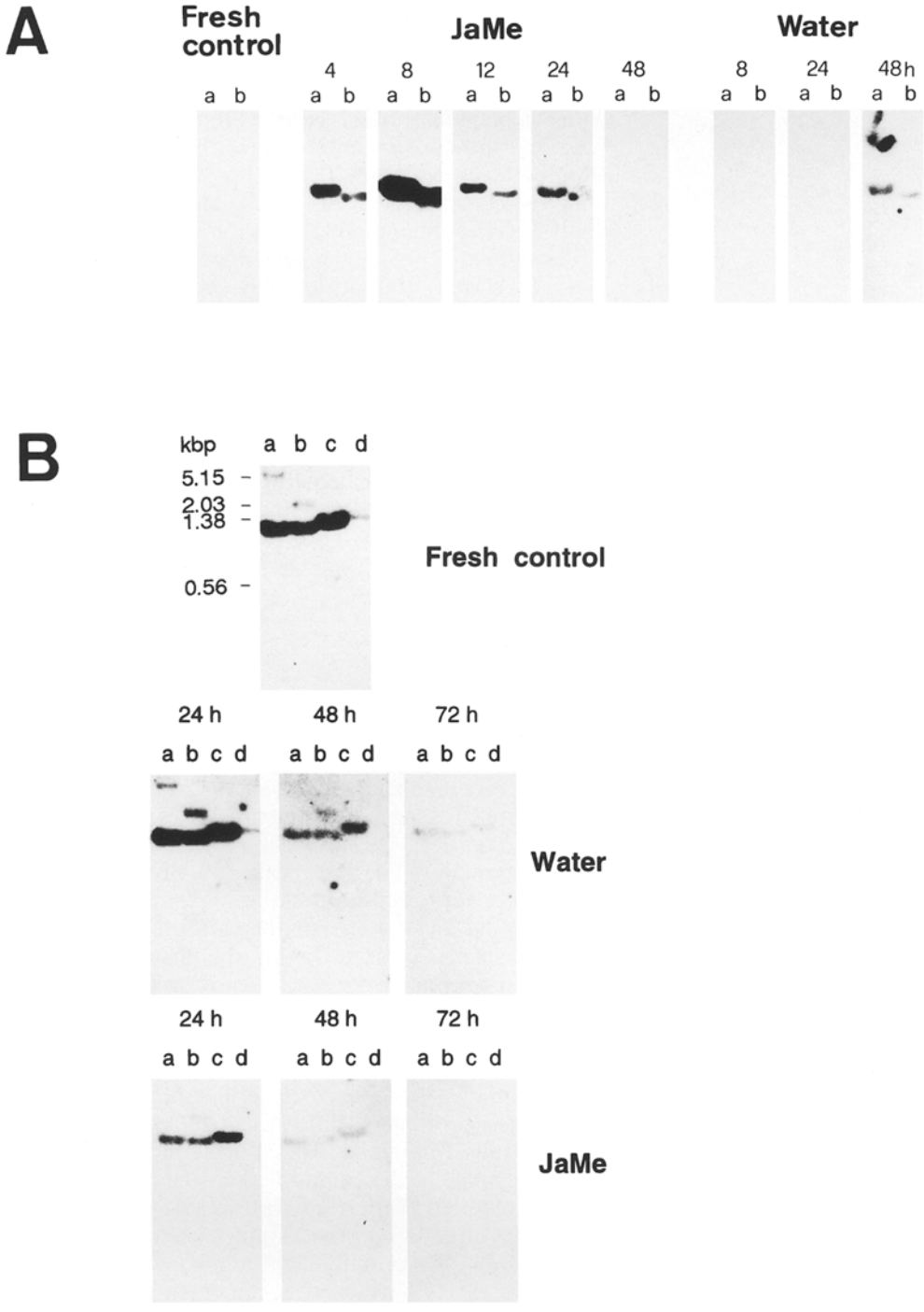

Fig. 1A,B. Transcription of *jip*, photosynthetic as well as actin and tubulin genes in JaMe- and water-treated barley leaf tissues. **A** *jip23-* (*lanes a*) and *jip15*-specific cDNA inserts (*lanes b*) were isolated, separated electrophoretically and transferred onto nitrocellulose filters, as described in *Materials and methods*. Filter-bound DNAs were hybridized to ^{32}P-RNAs synthesized by nuclei isolated from freshly harvested barley leaves or leaf tissues that had been treated with either JaMe or water for the indicated time periods. Nucleic acid hybrids were detected by autoradiography. **B** cDNA inserts complementary to mRNAs encoding the small subunit of Rubisco (*rbcS*; *lanes a*), a light-harvesting chlorophyll-*a*/*b*-binding protein (*lhbC1*; *lanes b*), actin (*lanes c*), and tubulin (*lanes d*) were hybridized to nuclear run-off transcripts, as described in **A**. The two high-molecular-mass bands hybridizing in some cases with the radiolabeled run-off transcripts (*lanes a* and *b*) likely represent incompletely digested plasmid DNAs

tained in both water- and JaMe-treated leaf tissues for the first 24 h of either treatment (Figs. 2, 3). Thereafter, the *rbcS* and *lhbC1* transcript levels displayed different decay kinetics. In JaMe-treated leaf tissues, the *rbcS* and *lhbC1* transcript levels were only slightly reduced during the next 12 h, but then, 36 h after the onset of JaMe treatment, rapidly declined (Fig. 3). In water-treated leaf tissues, the *rbcS* and *lhbC1* transcript levels dropped continuously during the second 24-h period (Figs. 2, 3), however.

To determine the half-life times of the *rbcS* and *lhbC1* transcripts in JaMe- and water-treated leaf tissues, inhibitor experiments were performed with cordycepin. This adenosine analogue blocks transcription by inhibiting incorporation of nucleotides into RNA (Cline and Rhem 1974).

In the first experiment, cordycepin was added to the leaf samples 24 h after the onset of water or JaMe treatment. As shown in Fig. 3 for water-treated leaf tissues, the *rbcS* and *lhbC1* transcripts were turned over with half-lives of approx. 6 and 3 h, respectively. In contrast, the half-life times of the *rbcS* and *lhbC1* transcripts initially appeared to be much higher in JaMe-treated leaf tissues than in water controls (Fig. 3). These

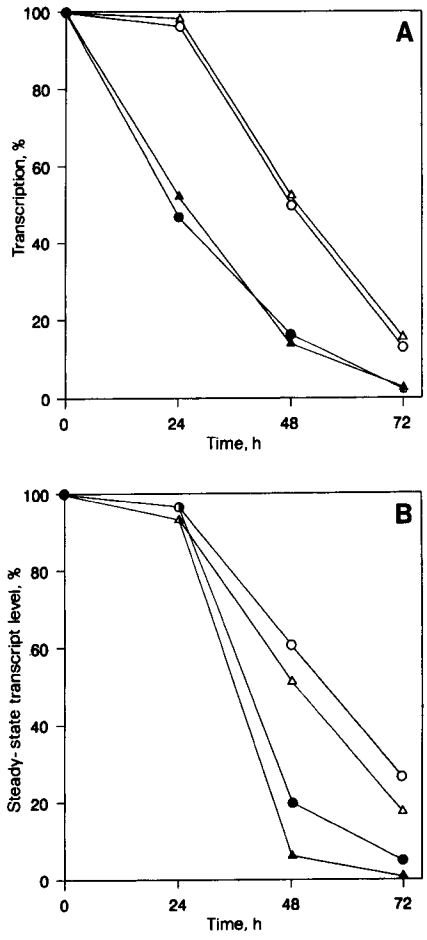

Fig. 2A,B. Transcription rates of *rbcS* and *lhbC1* and transcript levels in JaMe- and water-treated leaf tissues. **A** The rate of transcription of the *rbcS* and *lhbC1* genes was determined as described in Fig. 1. **B** Steady state transcript levels of *rbcS* and *lhbC1* were determined by Northern hybridization with cDNA inserts identical to those used for nuclear run-off assays. *rbcS* (△, ▲) and *lhbC1* (○, ●) transcript levels in JaMe- (●, ▲) and water-treated (○, △) leaf segments are expressed as percentages of the levels found in freshly harvested leaves, set as 100%

transcripts were rather stable, displaying half-lives of approx. 28 h (*lhbC1*) and 19.5 h (*rbcS*). After 36 h, however, the *rbcS* and *lhbC1* transcripts were rapidly degraded.

In order to precisely determine the kinetics of decay of the *rbcS* and *lhbC1* transcripts during later stages of the jasmonate response, cordycepin was added to leaf segments after 36 h of JaMe exposure. As calculated from the data shown in Fig. 3, the half-life times of the *rbcS* and *lhbC1* transcripts were 0.9 h and 1.5 h, respectively, indicating that each transcript was destabilized by a factor of approx. 18 during this period of JaMe treatment.

Determination of the overall rate of protein synthesis. By analogy to *jip15* and *jip23*, the gene encoding the previously identified 60-kDa ribosome-inactivating protein (JIP60; Becker and Apel 1992; Reinbothe et al. 1994c) was expected to be transcriptionally activated in JaMe-treated barley leaf tissues. To demonstrate this, the rate of *jip60* gene transcription was determined. In addition, we determined whether the time courses of JIP60 mRNA and protein accumulation were consistent with the previously observed general down-regulation of protein synthesis in JaMe-treated barley leaf tissues (Reinbothe et al. 1994c). To this end, Western blot analysis (Lehmann et al. 1995) and Northern blot analysis (Reinbothe et al. 1994c) were performed.

As shown in Fig. 4, the gene encoding JIP60 was in fact transiently transcriptionally activated (Fig. 4A, triangles). In turn, *jip60* mRNA accumulated (Fig. 4A, circles) and was subsequently translated into JIP60 protein (Fig. 4B, squares) which began to depress the overall rate of protein synthesis, as measured by determining the rate of translation initiation (Fig. 4B, arrows). Transcription of the *jip60* gene was also detectable in water-treated leaf tissues but to a much lower extent and at a later stage than in JaMe-treated leaf tissues, by analogy to transcription of *jip15* and *jip23* genes (cf. Fig. 1).

Discussion

Detached leaf segments of barley respond to JaMe with a characteristic temporal pattern of change in gene expression. At least four stages of the jasmonate response can be distinguished at this level. The first is a very rapid response of induction of cytoplasmic JIP synthesis, that, as we have shown here, is controlled transcriptionally. The *jip* genes in barley are first transcribed approx. 4 h after the onset of JaMe treatment (Figs. 1 and 4). Shortly thereafter, i.e. 4–8 h after exposing leaf tissues to JaMe, the first *jip* mRNAs appear. In contrast to *jip* genes, genes encoding proteins involved in photosynthesis were rapidly turned off after the onset of JaMe treatment. One may therefore conclude that *jip* and light-regulated genes of barley might contain shared, as well as distinct, *cis*-elements within their promoters that caused the observed differences in transcriptional control. Furthermore, one might speculate that JaMe caused either the formation of factors required for transcription of *jip* genes or inhibited synthesis of pre-existing proteins needed for transcription of normally light-regulated genes. Both jasmonate effects have been documented for numerous other polypeptides in barley leaf tissues (summarized by Reinbothe et al. 1994b).

During the second stage of the jasmonate response, beginning after approx. 8 h, most pre-existing (control) mRNAs should be displaced from polysomes in favor of *jip* mRNAs. Translation initiation is the site at which protein synthesis is selectively depressed by JaMe (Reinbothe et al. 1993a). After approx. 24 h of JaMe treatment, control but not JIP protein synthesis has ceased, although mRNAs such as *rbcS* and *lhbC1* are still present at pretreatment levels in vivo (Figs. 2, 3). Despite the drastic reduction in transcription of the *rbcS* and *lhbC1* genes, their corresponding transcripts are maintained, suggesting that such mRNAs may be

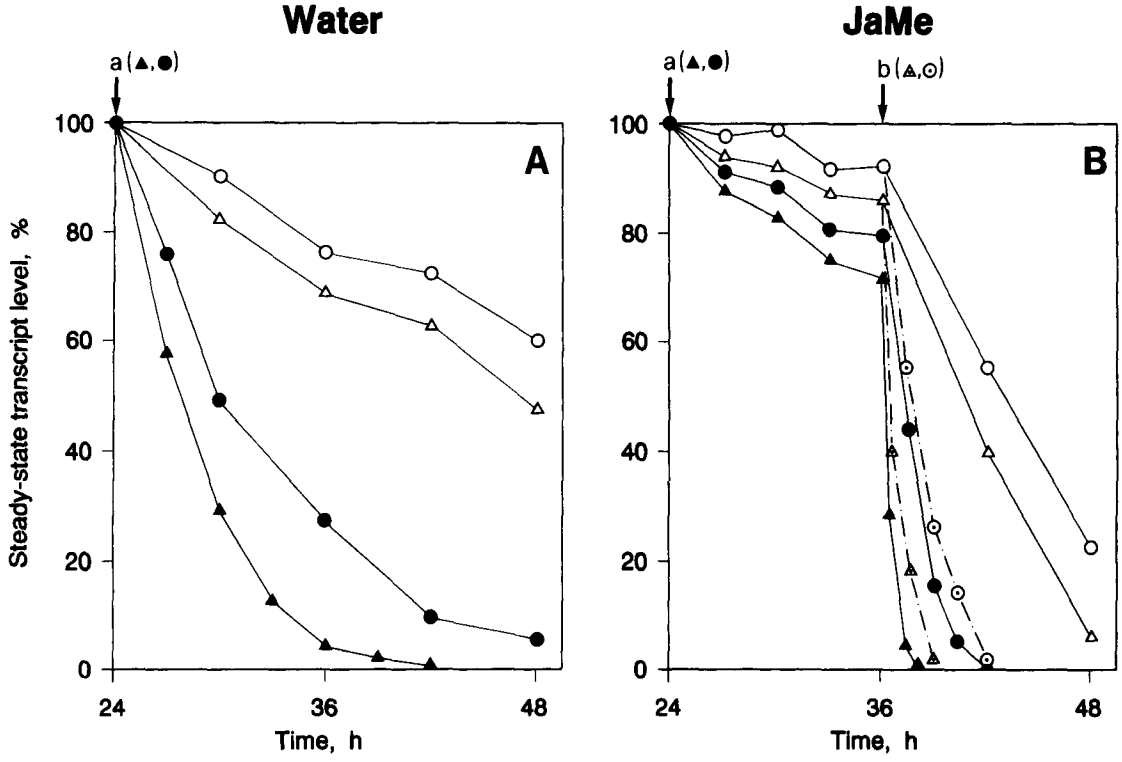

Fig. 3A,B. Effect of cordycepin on *rbcS* and *lhbC1* transcript abundances in water- (**A**) and JaMe-treated (**B**) leaf tissues. Steady state transcript levels of *rbcS* (△, ▲, ▲) and *lhbC1* (○, ⊙, ●) were determined by Northern hybridization of total RNA from water- and JaMe-exposed leaf segments that had been treated without (△, ○) or with cordycepin at t_{24} (▲, ●; *arrow a*) or t_{36} (▲, ⊙; *arrow b*), as described in Fig. 2B. Note that the *rbcS* and *lhbC1* transcript levels refer to those transcript levels that were determined in leaf tissues that had already been exposed to either water or JaMe for 24 h. At this time point, however, the *rbcS* and *lhbC1* transcript levels had dropped by approx. 8% and 5%, respectively, as shown in Fig. 2

preserved in specialized subcellular structures, such as messenger ribonucleoprotein particles (Larson and Sells 1987; Scherrer 1990), in JaMe-treated leaf tissues.

During the third stage of the jasmonate response, beginning after approx. 36 h, a massive decrease of control mRNAs, such as *rbcS* and *lhbC1*, can be observed (Figs. 2, 3). One may speculate that this rapid degradation of *rbcS* and *lhbC1* transcripts might be due to their release from the above-mentioned storage structures, such as messenger ribonucleoprotein particles. However, the half-lives of these transcripts appeared to be much shorter than those in water-treated leaf tissues, in which the levels of these transcripts also

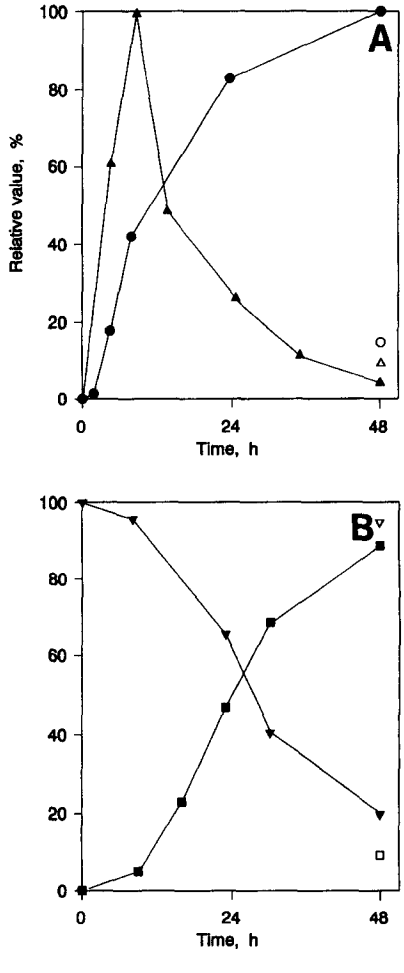

Fig. 4A,B. Expression of JIP60 in JaMe- (▲, ●, ■, ▼) and water-treated (△, ○, □, ▽) leaf tissues. **A** Changes in *jip60* gene transcription (△, ▲) and *jip60* steady-state transcript levels (○, ●) were determined as described in Fig. 2. **B** Concentrations of JIP60 protein (□, ■), determined by Western blotting, were compared with the overall rates of protein synthesis (▽, ▼). The latter was measured by analyzing polysome profiles of ribonucleoprotein material from the different leaf samples in sucrose step gradients and from there calculating the P/T ratios (as a measure of the proportion of polysomes to the sum of polysomes, ribosomal subunits and monosomes)

declined, suggesting that a selective mRNA destabilization occurs in JaMe-treated leaf tissues. It is as yet undetermined whether specific modifications in their 5′ and 3′ untranslated regions, which both have been shown to contain determinants for mRNA stability (summarized in Sachs 1993; Takagi et al. 1993), may destine *rbcS* and *lhbC1* transcripts to be degraded and whether this occurs by either constitutive or JaMe-induced ribonucleases. In contrast to photosynthetic transcripts, *jip* transcripts appeared to be stable for up to 48 h (Fig. 4).

Superimposed on these effects, a decay of cytoplasmic polysomes indicative of a general depression of translation initiation can be observed in long-term JaMe-treated leaf tissues (Fig. 4). This effect is likely due to the interaction of the previously identified ribosome-inactivating protein JIP60 with ribosomes marked specifically for this destination (Reinbothe et al. 1994b).

During the final stage of the jasmonate response, beginning after approx. 48 h, the cytoplasmic ribosomes and their ribosomal subunits, including their 25S and 18S rRNAs, decline drastically (Reinbothe et al. 1993b). In concert with changes in gene expression taking place in the plastid compartment (Reinbothe et al. 1993c), these alterations cause characteristic senescence symptoms, such as loss of chlorophyll and degradation of Rubisco and other chloroplast constituents, and ultimately lead to cell death.

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